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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Tom F. LUE, et al.

Serial No.: 09/909,544

Filing Date: July 19, 2001

For: METHODS AND COMPOSITIONS FOR PREVENTING AND TREATING MALE ERECTILE DYSFUNCTION AND FEMALE SEXUAL AROUSAL DISORDER

Examiner: Celine X. Qian

Group Art Unit: 1636

DECLARATION OF TOM F. LUE PURSUANT TO 37 C.F.R § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Tom F. Lue, in my individual capacity, hereby declares as follows:

1. I am one of the co-inventors of the above-referenced patent application, and am familiar with the contents thereof.

Bakircioglu

2. I have reviewed the publication entitled "The effect of adeno-associated virus-mediated brain-derived neurotrophic factor in an animal model for neurogenic impotence," published in *Journal of Urology*, Vol. 163, No. 4 Suppl., pp. 198 (April, 2000) (Bakircioglu), and cited by the Examiner, for which I am a co-author. The publication was cited by the Examiner as prior art against the application under 35 U.S.C. § 102(a).

3. The publication cited describes work that is encompassed by this patent application and its parent applications. The co-authors, Bakircioglu, M.E., Wefer, J., Sievert, KD and Fan, P., named in the publication were not involved in the conception of the subject matter of the presently claimed invention. Moreover, these co-authors worked under my and other co-inventors' direction performing work that led to the invention, but did not make an inventive contribution to the subject matter of the presently claimed invention.

Treatment of erectile dysfunction with vascular changes by BDNF

4. My co-inventors and I have performed, or have directed others to perform, experiments that show that a high fat diet caused erectile dysfunction with accompanying neurological and vascular changes. The changes include less nerve content, fewer endothelial cells and lighter smooth muscle content, hypermyelination and severe atrophy of axons, a remarkable decrease in the number and size of nonmyelinated axons, disarray of the smooth muscle cells with scant myofilaments and foamy cytoplasm and denuded endothelial lining of the sinusoids covered by numerous platelets (*See* Exhibit 1, Gholami et al., "The Effect of Vascular Endothelial Growth Factor and Adeno-Associated Virus Mediated Brian Derived Neurotrophic Factor on Neurogenic and Vasculogenic Erectile Dysfunction Induced by Hyperlipidemia," to be published in J. Urology (2003)). Our experiments also show that treatment with BDNF, *e.g.*, AAV-BDNF, alleviates, at least partially, these changes.

Prevention of erectile dysfunction by BDNF treatment

5. My co-inventors and I have performed, or have directed others to perform, experiments that show that, after nerve crash, BDNF/VEGF treatment can prevent and facilitate regeneration of nNOS containing neurons in the major pelvic ganglia, dorsal never and intracavernous tissue (*See* Exhibit 2. Bochinski et al., "The Effect of Vascular Endothelial Growth Factor and Brian Derived Neurotrophic Factor on Cavernous Nerve Regeneration in a Nerve-Crush Rat Model," manuscript submitted to British Journal of Urology). This result indicates that BDNF can have preventive, as well as therapeutic, effect to the erectile dysfunction.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

_____ _____

Date Tom F. Lue

EXHIBIT C

SN 09/909,544

ELSEVIER

DIAGNOSTIC EVALUATION OF THE ERECTILE FUNCTION DOMAIN OF THE INTERNATIONAL INDEX OF ERECTILE FUNCTION

JOSEPH C. CAPPELLERI, RAYMOND C. ROSEN, MICHAEL D. SMITH, AVANISH MISHRA, AND IAN H. OSTERLOH

ABSTRACT

Objectives. To evaluate the erectile function (EF) domain of the International Index of Erectile Function (IIEF) as a diagnostic tool to discriminate between men with and without erectile dysfunction (ED) and to develop a clinically meaningful gradient of severity for ED.

Methods. One thousand one hundred fifty-one men (1035 with and 116 without ED) who reported attempting sexual activity were evaluated using data from four clinical trials of sildenafil citrate (Viagra) and two control samples. The statistical program Classification and Regression Trees was used to determine optimal cutoff scores on the EF domain (range 6 to 30) to distinguish between men with and without ED and to determine levels of ED severity on the EF domain using the IIEF item on sexual intercourse satisfaction.

Results. For a 0.5 prevalence rate of ED, the optimal cutoff score was 25, with men scoring less than or equal to 25 classified as having ED and those scoring above 25 as not having ED (sensitivity 0.97, specificity 0.88). Sensitivity analyses revealed a robust statistical solution that was well supported with different assumed prevalence rates and several cross-validations. The severity of ED was classified into five categories: no ED (EF score 26 to 30), mild (EF score 22 to 25), mild to moderate (EF score 17 to 21), moderate (EF score 11 to 16), and severe (EF score 6 to 10). Substantial agreement was shown between these predicted and "true" classes (weighted kappa 0.80).

Conclusions. The EF domain possesses favorable statistical properties as a diagnostic tool, not only in distinguishing between men with and without ED, but also in classifying levels of ED severity. Clinical validation with self-rated assessments of ED severity is warranted. UROLOGY **54:** 346–351, 1999. © 1999, Elsevier Science Inc.

The International Index of Erectile Function (IIEF) has been shown to be a cross-culturally and psychometrically valid measure of male erectile dysfunction (ED).[1] The IIEF is a brief, reliable, and valid self-administered questionnaire of 15 questions (items). It contains five domains: (a) erectile function (six items), (b) orgasmic function (two items), (c) sexual desire (two items), (d) intercourse satisfaction (three items), and (e) overall sexual satisfaction (two items). The instrument has been used to measure efficacy for oral sildenafil in the treatment of ED[2] and for oral phentolamine in the treatment of minimal ED.[3]

Other scales exist for male sexual function.[4–6] We know of no widely accepted scale, however, that measures erectile function (EF) by means of a validated instrument that provides a single cutoff score for use as a simple diagnostic tool to help distinguish between men with and without ED. Moreover, no standard classification system exists that grades an individual's degree of severity of ED based on total EF scores. A severity classification of ED would be useful in comparing results of different trials using the EF domain of the IIEF and in monitoring changes in EF in a cohort of patients over time.

We address one of the recommendations of the National Institutes of Health (NIH) Consensus Development Conference on Impotence in calling for

From Pfizer Central Research, Groton, Connecticut; University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey; Pfizer Central Research, Sandwich, United Kingdom; and Pfizer Pharmaceuticals Group, New York, New York

Reprint requests: Joseph C. Cappelleri, Ph.D., M.P.H., Department of Clinical Research, Pfizer Central Research, Eastern Point Road, Groton, CT 06340-8030

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TABLE I. *Baseline characteristics of subjects*

	Men Included		
	Subjects with ED	Controls	Men Excluded (All Had ED)
Physical measures			
Age (yr)	55.75 ± 10.36 (n = 1035)*	54.07 ± 10.19 (n = 116)	58.29 ± 10.56 (n = 655)
Height (cm)	1.77 ± 0.073 (n = 1031)	1.76 ± 0.08 (n = 116)	1.77 ± 0.068 (n = 654)
Weight (kg)	84.78 ± 13.50 (n = 1030)	82.81 ± 10.97 (n = 116)	85.03 ± 13.39 (n = 655)
Body mass index (kg/m^2)	26.87 ± 3.81 (n = 1029)	26.82 ± 3.74 (n = 116)	27.07 ± 4.00 (n = 654)
Duration of ED (yr)	4.14 ± 4.29 (n = 1035)*	—	4.60 ± 4.61 (n = 655)
Race	n = 1035	n = 116	n = 655
White	97 (93.8)†	112 (96.5)	611 (93.3)
Black	39 (3.8)	0 (0.0)	25 (3.8)
Asian	8 (0.8)	1 (0.9)	8 (1.2)
Other	17 (1.6)	3 (2.6)	11 (1.7)
Primary etiology	n = 1035	—	n = 655
Organic	515 (49.8)†	—	348 (53.1)
Psychogenic	227 (21.9)	—	101 (15.4)
Both	290 (28.0)	—	200 (30.5)
Other	3 (0.3)	—	6 (1.0)
Cigarette smoking	n = 1035	n = 98	n = 655
Current smokers	268 (25.9)†	13 (13.3)	159 (24.3)
Exsmoker	451 (43.6)	44 (44.9)	325 (49.6)
Never smoked	316 (30.5)	41 (41.8)	171 (26.1)
Medications			
Antihypertensives	198 (21.7)‡	22 (19.0)	166 (28.2)
Analgesics	50 (5.5)	10 (8.6)	30 (5.1)
Antidepressants	34 (3.7)	1 (0.9)	29 (4.9)
Beta-blockers	43 (4.7)	7 (6.0)	34 (5.8)
Ulcer-healing	65 (7.1)	8 (6.9)	37 (6.3)
Diabetic	77 (8.5)	5 (4.3)	79 (13.4)

KEY: ED = erectile dysfunction.
Data presented as mean ± standard deviation, unless otherwise noted.
* *n = number of subjects who responded.*
† *Number of subjects (%); percentages may not equal 100% because of rounding error.*
‡ *Number of subjects (%) who responded taking a particular medication.*

studies of diagnostic assessments of ED and the development of a staging system to quantify the degree of ED.[7] The use of standardized scales for clinical decision making can also be incorporated into educational initiatives on ED, another recommendation of the NIH panel.[7] Therefore, we evaluated the utility of the EF domain of the IIEF as a diagnostic instrument.

MATERIAL AND METHODS

EF Domain

The six items on the EF domain include detailed questions concerning erection frequency, erection firmness, penetration ability, maintenance frequency, maintenance ability, and erection confidence.[1] All analyses were restricted to baseline (pretreatment) data. The enrolled samples in the analyses were composed of men who reported having had sexual activity at least once during the 4 weeks before their baseline responses to the questions. Each item was therefore based on a 5-point Likert scale. For each subject, the responses of all six items of the EF domain were summed to arrive at a total EF score, with a range from 6 to 30. A higher total score indicated relatively better erectile functioning.

Selecting Men With and Without ED

The diagnostic evaluation concentrated on discriminating between men with an established clinical diagnosis of ED and men not clinically diagnosed with ED. Subjects in the ED group participated in a total of four double-blind, placebo-controlled Phase III multicenter clinical trials of sildenafil (Viagra, Pfizer) for the treatment of ED. The criteria for inclusion required that men with ED be 18 years old or older, be in a stable and heterosexual relationship for at least the past 6 months, and have a clinical diagnosis of ED for at least 6 months as evaluated by their physician. Subjects with a broad-spectrum etiology (organic to psychogenic) were included. Subjects were excluded if they had penile anatomic defects, an uncontrolled major medical illness or psychological disorder, or known drug or alcohol dependence.

Subjects in the group without established ED (controls) were volunteers from two independent studies who were recruited from an outpatient community health center. They were without any history of ED and clinically judged by the primary investigator as having normal EF.

Of 1806 men who reported sexual activity, 1151 (63.7%) provided complete (nonmissing) responses to the six questions: 1035 had documented ED and 116 did not. Table I contains a summary of their baseline characteristics. The 655 subjects who omitted at least one of the six questions on the EF domain were excluded from the analysis (Table I). Table I shows that the baseline characteristics of the excluded sub-

jects, all with ED, were clinically comparable to those of the men with ED included in the analysis. Compared with indexes of ED constituting a single item or fewer than six items, the six-item index may increase the proportion of men unable to be classified due to item nonresponse.

Classifying Degrees of ED

No question was asked of men to self-rate their degree of ED, nor did clinicians rate the overall severity of ED of their patients. Since there was no independent criterion for degree of ED severity, we created a surrogate measure for it. Item 7 of the IIEF—on sexual intercourse satisfaction—was selected for three reasons. First, among items not on the EF domain, it was the most salient variable in discriminating between men with and without ED. Second, it was the most reliable predictor of EF scores, not only for the entire sample, but also for patients who were clinically diagnosed with ED. Third, satisfaction is an essential component of the NIH definition of ED.[7] The first two reasons were supplied by the statistical program Classification and Regression Trees (CART).[8–10] For the 1151 men with EF scores, 1135 (98.6%) gave a completed response on Item 7 and reported attempting sexual intercourse (in the past 4 weeks).

Statistical Analysis

Choosing an Optimal Cutoff. The statistical program CART[8–10] was employed to select optimal cutoff scores that "best" partitioned the binary outcome (ED, no ED) and to conduct cross-validation analyses. A complementary publication[8] gave an optimal cutoff score of 25 for the main analysis based on a prevalence rate of ED in a clinical population that is approximately equal to 0.50, a reasonable estimate that falls within the range of estimated prevalence rates cited in published reports.[11] An implication of this is that the error of misclassifying a man without ED is assumed to be the same, or as important, as that of misclassifying a man with ED.

In the present report, we extended our methodology by allowing for different assumed prevalence rates in the form of sensitivity analyses. Such analyses enhance the overall analysis and provide clinicians with additional diagnostic information about specific samples of patients whose set of risk factors may suggest prevalence rates of ED different from 0.5. For the sensitivity analyses, we set CART to have prevalence rates of ED equal to 0.20 through 0.65, in increments of 0.05, to reflect a likely range of prevalence rates of ED in a clinical sample.

Determining Grades of Severity. To establish a gradient of severity for ED, we adopted the following three-tier approach:

Step 1. The clinical outcome (ED, no ED) was expressed as a function of the five ordinal values on Item 7 (satisfaction with sexual intercourse) to decide which categories of Item 7 belong to men with ED and which belong to men without ED.

Step 2. For men with ED, categories of Item 7 that belonged to ED (from Step 1) were treated as ordinal responses and expressed as a function of EF scores to arrive at a range of scores that corresponded to each category.

Step 3. Using Scheffé's multiple comparisons test in the Statistical Analysis System,[12] we tested whether the mean EF score of a given category of ED significantly differed from each of the other categories.

The range of EF scores for no ED was determined by the aforementioned main analysis with the cutoff interval between 26 and 30 (inclusive) set beforehand as the range of EF scores for no ED.

Diagnostic Statistical Analysis. For the sensitivity analyses on individual cutoffs, several standard diagnostic statistics were obtained: sensitivity, specificity, positive and negative predictive value, kappa, and odds favoring ED.[13–15] For the analysis on ED severity, the weighted kappa coefficient[16,17] was calculated to provide an indication of overall agreement, beyond chance, between the "true" classification of ED severity (from categories on Item 7) and the predicted classification of ED severity (from EF scores). In addition, the correlation between these two sets of classifications were computed using the Spearman rank-order correlation and the Kendall rank-order (tau-b) correlation.[18] Exact 95% confidence intervals (CIs) of all diagnostic measures were computed by StatXact.[17]

RESULTS

Single Cutoff Scores

Table II shows the results for the different assumed prevalence rates of ED for the sample data. The optimal cutoff was 22 for prevalence rates of ED from 0.20 to 0.30, 25 for prevalence rates from 0.35 to 0.60, and 26 for a prevalence rate of 0.65. Diagnostic values for the optimal cutoff scores gave relatively high discrimination and, although the kappa values were lower for the lowest three prevalence rates, were generally similar across prevalence rates. All cross-validation results (not shown) were very similar to and supportive of the results from the sample data.

Moreover, the average total score on the EF domain was 14.37 (standard deviation [SD] 5.96, range 6 to 30) for subjects with documented ED and 28.14 (SD 3.31, range 11 to 30) for controls, a statistically significant difference ($P = 0.0001$).

Levels of ED Severity

Men who responded with a response of 1 (almost never/never), 2 (a few times [much less than half the time]), 3 (sometimes [about half the time]), or 4 (most times [much more than half the time]) to their satisfaction with sexual intercourse (Item 7 of the IIEF) were classified as having ED; those who responded with a code of 5 (almost always/always) were classified as not having ED (Step 1).

Hence, only men who responded 1, 2, 3, or 4 to Item 7 and who were clinically diagnosed with ED were included in the next step (Step 2). For the intercourse satisfaction item, the category "almost never/never" corresponded to an EF score between 6 and 10 (inclusive), "a few times" to a score between 11 and 16, "sometimes" to a score between 17 and 21, "most times" to a score between 22 and 25, and "almost always/always" to a score between 26 and 30. Thus, ED severity was labeled and classified into the following five categories: severe (EF score 6 to 10), moderate (EF score 11 to 16), mild to moderate (EF score 17 to 21), mild (EF score 22 to 25), and no ED (EF score 26 to 30).

Mean EF scores for men with ED increased, as expected, with increased satisfaction with intercourse: 9.80 (SD 4.09) for category 1 of Item 7; 13.76 (SD 4.11) for category 2; 17.75 (SD 4.24) for category 3; and 19.83 (SD 4.84) for category 4. The

TABLE II. *Diagnostic statistics for different prevalence rates: sample results*

Prevalence Rate of ED*	Optimal Cutoff	Sensitivity†	Specificity‡	Predicted Value		Kappa Coefficient¶	Posterior Odds Favoring ED Over No ED#
				Positive§	Negative‖		
0.20	22	0.89 (0.86, 0.90)**	0.93 (0.87, 0.97)**	0.76 (0.71, 0.81)**	0.97 (0.96, 0.98)**	0.57 (0.51, 0.64)**	3.17 (2.45, 4.26)**
0.25	22	0.89 (0.86, 0.90)	0.93 (0.87, 0.97)	0.81 (0.76, 0.85)	0.96 (0.95, 0.97)	0.57 (0.51, 0.64)	4.26 (3.17, 5.67)
0.30	22	0.89 (0.86, 0.90)	0.93 (0.87, 0.97)	0.84 (0.80, 0.88)	0.95 (0.93, 0.96)	0.57 (0.51, 0.64)	5.25 (4.00, 7.33)
0.35	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.81 (0.77, 0.85)	0.98 (0.97, 0.99)	0.78 (0.72, 0.84)	4.25 (3.35, 5.67)
0.40	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.84 (0.81, 0.87)	0.98 (0.96, 0.99)	0.78 (0.72, 0.84)	5.25 (4.26, 6.69)
0.45	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.87 (0.84, 0.89)	0.97 (0.96, 0.98)	0.78 (0.72, 0.84)	6.69 (5.25, 8.09)
0.50††	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.89 (0.86, 0.91)	0.97 (0.95, 0.98)	0.78 (0.72, 0.84)	8.09 (6.14, 10.11)
0.55	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.91 (0.88, 0.93)	0.96 (0.94, 0.97)	0.78 (0.72, 0.84)	10.11 (7.33, 13.28)
0.60	25	0.97 (0.95, 0.98)	0.88 (0.80, 0.93)	0.92 (0.90, 0.94)	0.95 (0.92, 0.97)	0.78 (0.72, 0.84)	11.50 (9.00, 15.67)
0.65	26	0.98 (0.97, 0.99)	0.85 (0.77, 0.91)	0.92 (0.90, 0.94)	0.95 (0.93, 0.98)	0.83 (0.77, 0.88)	11.50 (9.00, 15.67)

Key: ED = erectile dysfunction; EF = erectile function.

* *In this context, the ratio of the prevalence rates of no ED to ED gives the relative seriousness of misclassifying a subject without ED to misclassifying a subject with ED.*[9,10] *For instance, a prevalence rate of no ED equal to 0.8 implies that it is four times (=0.8/0.2) more serious to misclassify a subject without ED than to misclassify a subject with ED.*

† *Proportion of men with ED correctly classified as such by their EF scores.*

‡ *Proportion of men without ED correctly classified as such by their EF scores.*

§ *Proportion of men who did have ED among men classified as having ED.*

‖ *Proportion of men who did not have ED among men classified as not having ED.*

¶ *Measure of agreement, beyond chance, between true and predicted diagnoses (possible range −1 to 1).*

Frequency of ED to no ED in subjects who were classified as having ED (ie, those with a score less than or equal to a given cutoff) from a population with a given prevalence rate of ED.

** *Numbers in parentheses represent the exact 95% confidence interval.*

†† *Main analysis. In sensitivity analyses, subjects with a missing value on an item were assigned the median value of the completed responses to that item among men with ED and, separately, among men without ED. Results on sensitivity and specificity including these subjects were almost identical (sensitivity 0.98, specificity 0.88) to those found for subjects with completed scores.*

TABLE III. *Number of men classified by "true" grading and predicted grading for erectile dysfunction**

"True" Grading of ED	Predicted Grading of ED†					
	Severe	Moderate	Mild to Moderate	Mild	No ED	Total
Severe	239	77	18	7	1	342
Moderate	52	152	58	8	1	271
Mild to moderate	11	59	91	32	4	197
Mild	4	19	35	41	8	107
No ED	0	0	0	4	92	96
Total	306	307	202	92	106	1013

KEY: ED = erectile dysfunction; IIEF = International Index of Erectile Function; EF = erectile function.

* As specified in the text, a "true" grading was based on a category of intercourse satisfaction (Item 7 on the IIEF), and a predicted grading was based on a range of scores on the EF domain.

† These results are based on a prevalence rate of ED equal to 0.35 through 0.60, perhaps the most likely range of the true prevalence rate in a clinical population, in which the predictive severity of ED was classified into five categories: no ED (EF score 26–30), mild (EF score 22–25), mild to moderate (EF score 17–21), moderate (EF score 11–16), and severe (EF score 6–10). For a prevalence rate of ED equal to 0.65, the mild category would range from 22 to 26 (inclusive) and the no ED category would range from 27 to 30. For prevalence rates of ED between 0.2 and 0.3, the mild category (22 to 25) would be omitted and the category with no ED would have scores from 22 to 30.

mean was 29.01 (SD 1.59) for men without ED who responded to category 5. Each of the 10 pairs of five category means was significantly different according to Scheffé's 95% CIs (Step 3).

Table III provides the number of men classified by "true" grade and predicted grade of ED severity. Men were classified correctly into their "true" level of ED more often than any other level. Men who were misclassified tended to be assigned into a degree category adjacent to the "correct" category, rather than into a more remote category. Overall, substantial agreement between "true" and predicted grades was found; the weighted kappa value was 0.80 (95% CI 0.77 to 0.83). The Spearman and Kendall (tau-b) rank-order correlations were 0.75 (95% CI 0.71 to 0.79) and 0.68 (95% CI 0.65 to 0.72), respectively.

COMMENT

The CART algorithm, which we applied to establish optimal cutoff scores, has been extensively applied to a wide range of clinical decision aids in published reports.[9,10] A cutoff score does not need to be optimal, however, to have adequate diagnostic characteristics; several cutoffs may be appropriate.[8–10]

We used only the EF domain of the IIEF to diagnose the presence and severity of ED. In general, favorable results were obtained that were easily interpreted. When the risk factors in Table I were added, they did not contribute to the final model.

If men who reported no sexual activity are considered, we suggest that the category of severe ED be 1 to 10, instead of 6 to 10, on the EF domain, provided that they were involved in a stable relationship with a female partner. When these men were added to the analysis, the same level of agreement between predicted and "true" levels of ED was obtained (weighted kappa 0.80).

Our analysis on ED severity included severe, moderate, mild to moderate, mild, and no ED in accordance with our clinical understanding of the scores on the EF domain. Other investigators[19] have labeled classes of ED on the EF domain in the same way as a clinically suitable classification of men who are neither strictly mild nor strictly moderate in their degree of ED.

Future research is needed on the validity of our severity classification for ED. This research should include a validation study that assesses the degree of agreement and magnitude of correlation between subject self-assessment of EF and the EF domain of the IIEF with respect to levels of ED severity.

CONCLUSIONS

These data support the validity of the EF domain of the IIEF as a diagnostic tool in clinical settings for grading degrees of severity of ED and for distinguishing between men with and without the disorder. The IIEF is currently widely used in clinical trials of ED; the proposed classification would potentially enhance its use in both research and clinical settings. The proposed classification system, however, should not be viewed as a perfect diagnostic discriminator. The classifications are intended to enhance the decision making of clinicians who are likely to perform more detailed evaluations in individual cases. The results of our investigation are highly encouraging in this regard and warrant further research.

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REFERENCES

1. Rosen RC, Riley A, Wagner G, *et al:* The International Index of Erectile Function (IIEF): a multidimensional scale for assessment of erectile dysfunction. Urology 49: 822–830, 1997.

2. Goldstein I, Lue TF, Padma-Nathan H, *et al,* for the Sildenafil Study Group: Oral sildenafil in the treatment of erectile dysfunction. N Engl J Med 338: 1397–1404, 1998.

3. Goldstein I, and the Vasomax Study Group: Efficacy and safety of oral phentolamine (Vasomax) for the treatment of minimal erectile dysfunction. J Urol 159(suppl): 240, 1998.

4. Feldman HA, Goldstein I, Hatzichristou DG, *et al:* Impotence and its medical and psychosocial correlates: results of the Massachusetts male aging study. J Urol 151: 54–61, 1994.

5. Feldman HA, Goldstein I, Hatzichristou DG, *et al:* Construction of a surrogate variable for impotence in the Massachusetts Male Aging Study. J Clin Epidemiol 47: 457–467, 1994.

6. O'Leary MP, Fowler FJ, Lenderking WR, *et al:* A brief male sexual function inventory for urology. Urology 46: 697–706, 1995.

7. NIH Consensus Development Panel on Impotence: Impotence. JAMA 270: 83–90, 1993.

8. Cappelleri JC, Rosen RC, Smith MD, *et al:* Some developments on the International Index of Erectile Function (IIEF). Drug Inf J 33: 179–190, 1999.

9. Steinberg D, and Colla P: *CART: Tree-Structured Nonparametric Data Analysis.* San Diego, Salford Systems, 1995.

10. Breiman L, Friedman J, Olshen R, *et al: Classification and Regression Trees.* Pacific Grove, Wadsworth, 1984.

11. Benet AE, and Melman A: The epidemiology of erectile dysfunction. Urol Clin North Am 22: 699–709, 1995.

12. SAS Institute: *SAS/STAT® User's Guide, version 6,* 4th ed. Cary, North Carolina, SAS Institute, 1990, vol 2, p 944.

13. Weinstein MC, Fineberg HV, Elstein AS, *et al: Clinical Decision Analysis.* Philadelphia, WB Saunders, 1980, pp 75–130.

14. Dawson-Saunders B, and Trapp RG: *Basic & Clinical Biostatistics,* 2nd ed. Norwalk, Connecticut, Appleton & Lange, 1994, pp 232–248.

15. Cohen J: A coefficient of agreement of nominal scales. Educ Psychol Meas 20: 37–46, 1960.

16. Cohen J: Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit. Psychol Bull 70: 213–220, 1968.

17. *StatXact 3 for Windows: Statistical Software for Exact Nonparametric Inference User Manual.* Cambridge, CYTEL Software Corporation, 1995, pp 399–401, 639–663, 675–680.

18. Siegel S, and Castellan NJ: *Nonparametric Statistics for the Behavioral Sciences,* 2nd ed. New York, McGraw-Hill, 1988, pp 235–254.

19. Lamm S, and Couzens GS: *The Virility Factor.* New York, Simon & Schuster, 1998, p 98.

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THE EFFECT OF VASCULAR ENDOTHELIAL GROWTH FACTOR AND ADENO-ASSOCIATED VIRUS MEDIATED BRAIN DERIVED NEUROTROPHIC FACTOR ON NEUROGENIC AND VASCULOGENIC ERECTILE DYSFUNCTION INDUCED BY HYPERLIPIDEMIA

SHAHRAM S. GHOLAMI, RODMAN ROGERS, JOHNNY CHANG, HOA-CHUNG HO, TULIO GRAZZIOTTIN, CHING-SHWUN LIN AND TOM F. LUE*

From the Department of Urology, University of California, San Francisco, California

ABSTRACT

Purpose: We examined neurogenic and vasculogenic erectile dysfunction associated with hypercholesterolemia and evaluated vascular endothelial growth factor (VEGF) and adeno-associated virus (AAV) mediated, brain derived neurotrophic factor (BDNF) for potential treatment.

Materials and Methods: A total of 21, 2-month-old male rats were fed a 2% cholesterol diet and another seven were fed a normal diet. Two months later serum cholesterol levels were measured and test agents were given intracavernously. Those on normal diet (controls) received phosphate buffered saline (PBS). Those on cholesterol diet were randomly divided into 3 groups receiving PBS, VEGF (4 μg.) or AAV-BDNF (10^{10} viral particles). Four months later erectile function was evaluated and cavernous tissues were collected for erectile dysfunction and immunohistochemical staining.

Results: Serum cholesterol levels were higher in rats fed the high fat diet than in controls. Intracavernous pressure was lower in cholesterol plus PBS treated rats than in rats of the other 3 groups. All hypercholesterolemic rats had less nerve content, fewer endothelial cells and higher smooth muscle content than rats with normal cholesterol levels. In cholesterol plus PBS treated rats electron microscopy showed hypermyelination and severe atrophy of axons, a remarkable decrease in the number and size of nonmyelinated axons, disarray of the smooth muscle cells with scant myofilaments and foamy cytoplasm, and denuded endothelial lining of the sinusoids covered by numerous platelets. VEGF and AAV-BDNF appeared to alleviate partially these changes.

Conclusions: A high fat diet caused erectile dysfunction with accompanying neurological and vascular changes. VEGF and AAV-BDNF seemed to alleviate these problems.

KEY WORDS: impotence; rats, Sprague-Dawley; hyperlipidemia; endothelial growth factors; brain-derived neurotrophic factor

Erectile dysfunction affects as many as 30 million men in the United States with hypercholesterolemia as a contributing factor.[1] Hypercholesterolemia has been shown in rabbits to cause corporeal smooth muscle dysfunction and deficits in intracavernous blood flow, expandability and pressure decay.[2-4] At our laboratory it has previously been reported that hypercholesterolemia is associated with erectile dysfunction in rats.[5] Electron microscopy and histology of these rats showed damages to endothelial cells, intracavernous smooth muscle, and dorsal and intracavernous nerves. Therefore, it appears that hypercholesterolemia causes erectile dysfunction by affecting not only smooth muscle, but also penile nerves and blood vessels. To examine these possibilities and assess possible treatments we reestablished our hypercholesterolemic rat model and treated these rats with vascular endothelial growth factor (VEGF) or adeno-associated virus (AAV)-brain derived neurotrophic factor (BDNF) in the current study. The rationale for using VDGF and AAV-BDNF has been discussed in our previous reports.[6-8]

MATERIALS AND METHODS

Animal groups. A total of 28 male 2-month-old Sprague-Dawley rats were used in this study. All animal care, treatments and procedures were approved by the committee on animal research at our institution. Seven rats fed a normal diet served as controls. The remaining 21 rats were fed a 2% cholesterol diet. At 2 months, all rats underwent evaluation of serum cholesterol and were subsequently divided into 4 groups for intravenous treatments. Controls received (PBS), the cholesterol plus PBS group received PBS, the cholesterol plus VEGF group received 4 μg. VEGF and the cholesterol plus BDNF group received 10^{10} AAV-BDNF viral particles. Four months after these treatments all rats underwent erectile function testing, and measurement of serum cholesterol and systemic blood pressure. They were then sacrificed and penile tissues were harvested for electron microscopy and histological examination.

Intracavernous injection. A 1.5 cm. oblique incision was made in the lower abdominal midline. The skin was dissected from the anterior surface of the penis and the penis was retracted anterior with the foreskin left intact. Using blunt dissection the penile base and crura were exposed. The ischiocavernous muscles were dissected off of the anterior surface of the crus until the white of the tunica albuginea was identified. The crus was then gently cannulated using a 23 gauge butterfly needle. VEGF protein (Calbiochem, Inc., La Jolla, California) was administered at a dose of 4 μg. per injection in 0.1 ml. PBS with 0.1% bovine serum albumin. AAV-BDNF, which has been described previously,[6] was ad-

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ministered at 10^{10} viral particles per injection in 0.1 ml. PBS. Following injection the needle was left in place for 5 minutes and then removed to allow the medication to diffuse throughout the cavernous space. Immediately thereafter pinpoint electrocautery was applied to the needle hole for hemostasis, the wound was closed and the animal was allowed to recover under a heating pad.

Functional evaluation. Rats were anesthetized with intraperitoneal injection of pentobarbital (40 mg./kg.). A midline lower abdominal incision was made and the cavernous nerve was isolated bilaterally. The penile crura were exposed by removing part of the overlying ischiocavernous muscle. Electrostimulation was performed with a stainless steel bipolar hook electrode attached to a multijointed clamp. Monophasic rectangular voltage pulses converted to current pulses of 1.5 mA. with a frequency 20 Hz. at a pulse width of 0.2 milliseconds were delivered to each cavernous nerve for 50 seconds. A 23 gauge [redacted] was inserted into the right crus and connected to a pressure monitor. Arterial blood pressure was continuously monitored from a catheter inserted into the internal carotid artery.

A second 23 gauge needle was inserted into the left crus and papaverine (1 mg. in 0.1 ml.) was administered. After 10 minutes heparinized saline was instilled into the corpora at an incremental rate (0.01 to 0.1 ml. per second) to find the infusion rate necessary to maintain an intracorporeal pressure of 100 cm H_2O. The rate of pressure decrease after the termination of infusion was also measured.

Electron microscopy. Penile samples were immersion fixed in 2% glutaraldehyde and 0.2 M. sodium phosphate buffer, pH 7.4, overnight. After fixation in 2% aqueous osmium tetroxide for 2 hours the tissues were dehydrated in graded ethanol and propylene oxide, and subsequently embedded in Epon 812. Thick sections (1 μm.) were cut on a [redacted] microtome, stained with 1% methylene blue and examined for the best structural preservation under light microscopy. Thin sections (approximately 900 Å.) were mounted on 200-mesh copper grids and stained with 10% uranyl acetate and lead citrate as contrasting agents. Ultrastructural examination was performed with a Model 10 (Carl Zeiss, Jena, Germany) transmission electron microscope. At 20,000× magnification the dorsal nerve, intracavernous smooth muscle and endothelial cells were examined.

Histology. Paraffin sections (4 μm.) were hydrated and stained with antismooth muscle α-actin antibody (Sigma Chemical Co., St. Louis, Missouri). Images were viewed with a computer. Image analysis was done using Photoshop 6.0 (Adobe, San Jose, California) software and expressed as the total area of muscle or brownish areas in $\mu m.^2$. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining was done to assess the expression of nitric oxide synthase (NOS) containing nerves. Paraffin sections were cut at 10 μm., hydrated and incubated in 0.1 mM. NADPH, 0.2 mM. nitroblue tetrazolium and 0.2% Triton X-100 in 0.1 M. PO_4 for 60 minutes. NADPH diaphorase positive nerve tissues stained blue. Blue stained areas were calculated with computer image analysis as described and the mean areas positive per animal were compared between each treatment group. Endothelial cell staining was done on paraffin sections using antirat RECA-1 antibody.[9]

Statistical analysis. We used computer software[10] for statistical analysis. The data were first analyzed by 1-way ANOVA. If the difference was significant, the Student-Neuman-Keuls test was used to perform the pairwise comparisons.

RESULTS

Physiological parameters. The serum cholesterol levels of animals fed the 2% cholesterol diet were significantly higher than that of controls at 4 weeks and 6 months. Systemic arterial blood pressure was not significantly different among the 4 groups (table 1).

TABLE 1. *Anatomical parameters*

Groups	Mean Serum Cholesterol ± SD (mg./dl.) 4 Wks.	6 Mos.	Mean Blood Pressure ± SD (mm. Hg)
Controls	81 ± 8*	68 ± 10*	110 ± 8
Cholesterol + PBS	207 ± 25	811 ± 16	106 ± 14
Cholesterol + AAV-BDNF	224 ± 19	227 ± 18	102 ± 16
Cholesterol + VEFG	203 ± 13	236 ± 21	112 ± 11

* Versus other groups $p < 0.05$.

Functional studies. During electrostimulation of the cavernous nerve mean peak sustained intracavernous pressure ± SD was significantly lower in the cholesterol plus PBS group (41 ± 17 cm. H_2O) compared with the control (110 ± 12 cm. H_2O), cholesterol plus VEFG (91 ± 21 cm. H_2O) and cholesterol plus AAV-BDNF (97 ± 11 cm. H_2O) groups (table 2). After papaverine injection the cholesterol plus PBS group had significant lower intracavernous pressure than the other 3 groups. The normal diet control group had slightly higher intracavernous pressures than BDNF and VEGF treated animals but it did not reach statistical significance. No difference was noted with respect to infusion cavernosometry assessment of venous leakage when comparing controls and treated groups.

Histology. All hypercholesterolemic rats had significantly lower nerve content than normal diet control rats, suggesting that cholesterol may adversely affect nerve content/function (table 3). In our experience the neuronal NOS antibody stain and NADPH diaphorase stain are almost identical and, therefore, findings indicate a reduction in neuronal NOS containing nerves in the dorsal nerve. We also found a significantly decreased amount of endothelial cells in the cholesterol groups compared with normal diet control rats. Again, this observation suggests a decrease in endothelial cell content/function in these cholesterol treated animals. Conversely we noted higher smooth muscle content in hypercholesterolemic animals than in normal diet controls, although significant difference was noted only between AAV-BDNF and normal diet controls. Except for nerve content there was no significant difference in the VEGF and AAV-BDNF treated groups in regard to endothelial cell and smooth muscle content.

Electron microscopy. The dorsal nerve in normal diet control rats was filled with myelinated and nonmyelinated nerve bundles. In general, the axons were similar in size with little difference between the largest and smallest fibers. Individual nonmyelinated axons were also quite similar in size (fig. 1, *A*). The nuclei of Schwann cells were seen occasionally near the axons. In cholesterol plus PBS rats various degree of changes in the nerve fibers were noted (fig. 1, *B*). In some nerves only a mild decrease in axon size was noted. In others hypermyelination with severe atrophy of axons and marked difference in the sizes of the myelinated axons were noted. There was also a remarkable decrease in the number and size of nonmyelinated axons. In cholesterol plus VEGF rats myelinated axons were much larger than in cholesterol plus

TABLE 2. *Functional studies*

Groups	Mean Cavernous Stimulation ± SD (cm. H_2O)	Mean Papaverine Injection ± SD (cm. H_2O)	Maintenance Rate (cc/min.)
Controls	110 ± 12	118 ± 10	0.01
Cholesterol + PBS	41 ± 17*	58 ± 8*	0.025 ± 0.01
Cholesterol + AAV-BDNF	97 ± 11	116 ± 6	0.01
Cholesterol + VEFG	91 ± 21	109 ± 10	0.01

* Versus other groups $p < 0.05$.

TABLE 3. *Histochemistry on image analysis*

Groups	Mean Nerve ± SD (μmm.²)	Mean Smooth Muscle ± SD (μmm.²)	Mean Endothelium ± SD (μmm.²)
Controls	71.69 ± 86.26*	77,867 ± 26,976	1,310 ± 1,090*
Cholesterol + PBS	27.42 ± 19.34	128,944 ± 44,609	779 ± 620
Cholesterol + AAV-BDNF	92.49 ± 22.62†	151,840 ± 17,912‡	881 ± 722
Cholesterol + VEFG	14.52 ± 16.67	121,197 ± 61,469	779 ± 442

* Versus other groups $p < 0.05$.
† Significantly different versus cholesterol plus VEGF.
‡ Versus control $p < 0.05$.

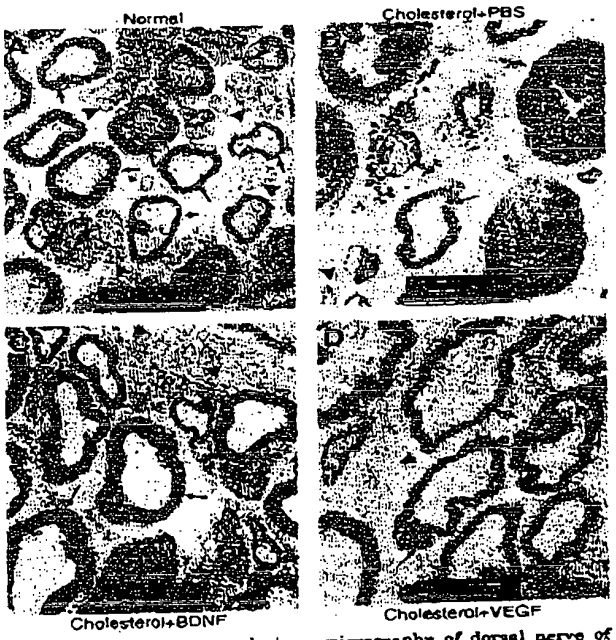


FIG. 1. Representative electron micrography of dorsal nerve of normal diet control (*A*), cholesterol plus PBS (*B*), cholesterol plus AAV-BDNF (*C*) and cholesterol plus VEGF (*D*) treated rats. Note myelin sheath (dark circles) and nonmyelinated nerves (small circles). Hypermyelination with atrophic axons (arrows) was noted in cholesterol plus PBS rats. Arrowheads indicate

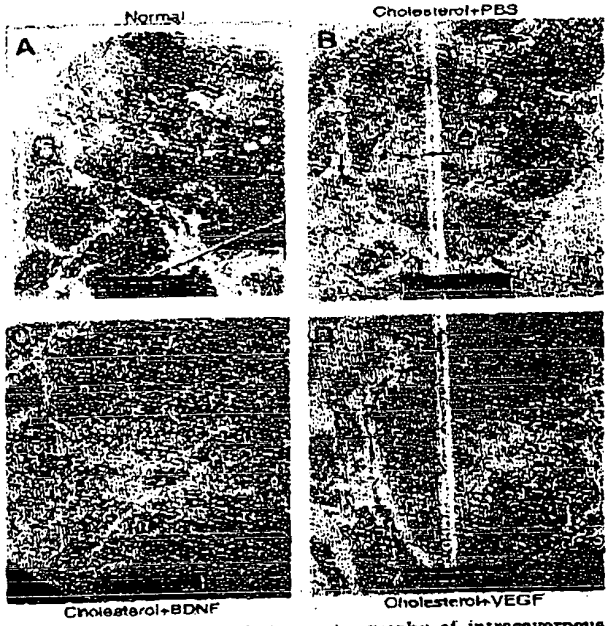


FIG. 2. Representative electron micrography of intracavernous smooth muscle of normal diet control (*A*), cholesterol plus PBS (*B*), cholesterol plus AAV-BDNF (*C*) and cholesterol plus VEGF (*D*) treated rats. Many foamy cells (arrows) with empty spaces and scant myofilaments were noted in cholesterol plus PBS rats.

PBS rats (fig. 1, *D*). There was also an increase in the number of nonmyelinated axons compared with cholesterol plus PBS rats. In hypercholesterolemic rats treated with AAV-BDNF myelinated and nonmyelinated axons appeared similar to those in normal diet control rats in size and number (fig. 1, *C*).

In regard to intracavernous smooth muscle in normal diet control rats smooth muscle cells, of which most were arranged in clusters, were embedded in fine strands of fibrous connective tissue (fig. 2, *A*). The cytoplasm of these myocytes contained abundant contractile myofilaments and dense bodies. Occasionally small aggregates of organelles, including mitochondria, rough endoplasmic reticulum and Golgi apparatus, were found adjacent to the nucleus. The cell membrane (sarcolemma) consisted of many alternating dense and light bands with the latter containing numerous pinocytotic vesicles (caveolae). The intercellular spaces were narrow and many cell-cell contacts (gap junctions) were visible. Nerve terminal varicosities were seen occasionally near clusters of smooth muscle cells. In cholesterol plus PBS treated rats many smooth muscle cells appeared to contain scant myofilaments and abundant empty space within the cytoplasm (fig. 2, *B*). In cholesterol plus AAV-BDNF and cholesterol plus VEGF treated rats most smooth muscle cells appeared normal with large amount of myofilaments and narrow intercellular spaces (fig. 2, *C* and *D*).

In the penis numerous endothelial cells line the sinusoids and blood vessels. In normal diet control rats the cavernous sinusoids and capillaries were lined with intact endothelium, of which the cytoplasm contained numerous pinocytotic vesicles (caveolae), mitochondria, rough endoplasmic reticulum and Golgi apparatus (fig. 3, *A*). The nuclei of endothelial cells were sparsely seen and appeared flattened. In cholesterol plus PBS rats many sinusoids were denuded of endothelium and lined by numerous platelets (fig. 3, *B*). In the capillaries thickened endothelium (intima hyperplasia) was often noted. Similar images were seen in rats treated with AAV-BDNF, although to a lesser degree (fig. 3, *C*). However, in hypercholesterolemic plus VEGF treated rats an increase in the number and size of endothelial nuclei was noted without thickened capillary endothelium or platelet adherence (fig. 3, *D*).

DISCUSSION

Although hypercholesterolemia induced erectile dysfunction has been extensively studied, we elected to reexamine this health problem in a rat model in an attempt to under-

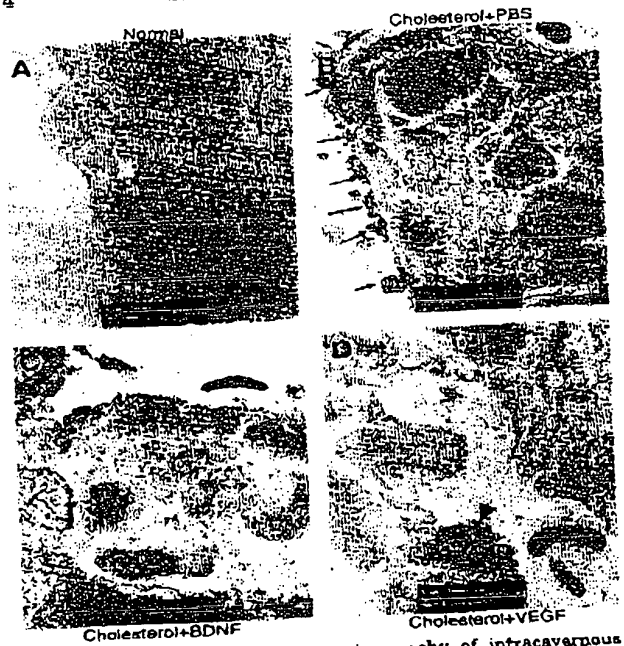


FIG. 3. Representative electron micrography of intracavernous erectile tissue of normal diet control (*A*), cholesterol plus PBS (*B*), cholesterol plus AAV-BDNF (*C*) and cholesterol plus VEGF (*D*) treated rats. Denuded endothelial lining with many attached platelets (arrows) was noted in cholesterol plus PBS group with similar but less prominent changes in cholesterol plus AAV-BDNF group. Large endothelial cell nucleus (arrow) was noted in cholesterol plus VEGF rats.

stand better the mechanism of the disease and explore alternative methods of treatment. Unlike previous studies that focused on smooth muscle and endothelial cell dysfunction to our knowledge the current study is the first to note also neurological alterations in hypercholesterolemia induced erectile dysfunction.

Because this study was designed to study the local effect of growth factor treatment and gene therapy on the penis, we did not expect these therapies to affect systemic cholesterol levels. Indeed, we found that all animals fed the high cholesterol diet had hypercholesterolemia regardless of the treatment given. Of interest is the lack of the effect of hypercholesterolemia on systemic blood pressure, suggesting that hypertension is not a likely contributing factor to erectile dysfunction in rats despite the frequent statement that hypertension is a factor contributing to erectile dysfunction in men.[11] Thus, since cholesterol levels and blood pressure were the same in all treated groups, the beneficial effects of AAV-BDNF and VEGF on erectile function were most likely due to local changes in penile tissue.

Interestingly there was a trend toward increased smooth muscle content in hypercholesterolemic rats. Since vascular smooth muscle cell proliferation is a pathological consequence of hypercholesterolemia,[12] it appears that increased cavernous smooth muscle content is also a manifestation of the diseased state of hypercholesterolemia. If so, the increased muscle content would reflect a change from the normal contractile state to the abnormal synthetic state of smooth muscle, thereby, contributing to erectile dysfunction. Treatment with VEGF or BDNF may have helped maintain larger degrees of the contractile properties of smooth muscle than in controls.

At the ultrastructural level we noted many foamy muscle cells with scant myofilaments or organelles in cholesterol plus PBS treated specimens. This finding is in agreement with the electron microscopic findings in hypercholesterolemic rabbits of Kim et al.[13] On the other hand, the morphology of most smooth muscle cells in AAV-BDNF and VEGF treated rats were similar to those in normal diet controls. Thus, these observations further support our contention that VEGF and BDNF may help maintain the contractile properties of cavernous smooth muscle cells under systemic hypercholesterolemia.

Electron microscopy also revealed remarkable alterations of myelinated and nonmyelinated nerve fibers in cholesterol plus PBS treated rats. This result could have been due to hyperlipidemia alone or to its associated reduction of the blood supply to the nerve. The partial restoration of myelinated and nonmyelinated nerve fibers in number and size in VEGF treated rats further illustrated neurotrophic effects of VEGF that we have reported previously.[7,8] However, as can be expected from a bona fide neurotrophic factor, BDNF treated rats had the most normal looking nerves of the 3 treated groups.

Examination of endothelial cells lining the sinusoids and capillaries also yielded several interesting results. Two striking findings were noted in cholesterol plus PBS cholesterol plus AAV-BDNF rats (to a lesser degree), namely denudation of endothelium and platelet attachment in many sinusoids, and thickening of endothelium in the capillaries. Denudation of endothelium impairs smooth muscle relaxation due to a lack of endothelial derived relaxation factor, which includes endothelial nitric oxide.[14] Platelet adhesion and attraction of monocytes and macrophages predispose the sinusoids to thrombus formation as well as to atherosclerosis.[15] In addition to thickening of the intima, these changes may reduce microperfusion of the smooth muscle and nerves and, thus, induce neurogenic and vasculogenic erectile dysfunction. In VEGF treated rats hypertrophy (with large nuclei) and hyperplasia (with more abundant nuclei) was seen in the sinusoidal and capillary endothelium. This finding could be an indirect evidence of angiogenesis and may explain improved erectile function in this group of rats.

CONCLUSIONS

In addition to the previously known effect of hypercholesterolemia on endothelium and blood vessels, we noted a detrimental effect of hypercholesterolemia on myelinated and nonmyelinated nerves of the penis. Hypercholesterolemia also caused a reduction of in NOS containing nerve and endothelial content but it appears to cause an increase in smooth muscle content. Intracavernous injection of AAV-BDNF preserved erectile function. This effect appears to be due to qualitative but not to quantitative preservation of nerves and smooth muscles. Intracavernous injection of VEGF protein has less protective effect on the nerve but seems to preserve the morphology of endothelial cells and smooth muscle through its presumed angiogenic effect.

REFERENCES

1. Wei, M., Macera, C. A., Davis, D. R., Hornung, C. A., Nankin, H. R. and Blair, S. N.: Total cholesterol and high density lipoprotein cholesterol as important predictors of erectile dysfunction. Am J Epidemiol, 140: 980, 1994
2. Azadzoi, K. M. and Saenz de Tejada, I.: Hypercholesterolemia impairs endothelium-dependent relaxation of rabbit corpus cavernosum smooth muscle. J Urol, 146: 238, 1991

3. Azadzoi, K. M., Park, K., Andry, C., Goldstein, I. and Siroky, M. B.: Relationship between cavernosal ischemia and corporal veno-occlusive dysfunction in an animal model. J Urol, 157: 1011, 1997
4. Nehra, A., Azadzoi, K. M., Moreland, R. B., Pabby, A., Siroky, M. B., Krane, R. J. et al: Cavernosal expandability is an erectile tissue mechanical property which predicts trabecular histology in an animal model of vasculogenic erectile dysfunction. J Urol, 159: 2229, 1998
5. Bakircioglu, M. E., Hsu, K., El-Sakka, A., Sievert, K.-D., Lin, C. S. and Lue, T. F.: Effect of a Chinese herbal medicine mixture on a rat model of hypercholesterolemic erectile dysfunction. J Urol, 164: 1798, 2000
6. Bakircioglu, M. E., Lin, C.-S., Fan, P., Sievert, K.-D., Kan, Y. W. and Lue, T. F.: The effect of adeno-associated virus mediated brain derived neurotrophic factor in an animal model of neurogenic impotence. J Urol, 165: 2108, 2001
7. Lee, M.-C., El-Sakka, A. I., Graziottin, T. M., Ho, H.-C., Lin, C.-S. and Lue, T. F.: The effect of vascular endothelial growth factor on a rat model of traumatic arteriogenic erectile dysfunction. J Urol, 167: 761, 2002
8. Rogers, R. S., Graziottin, T. M., Lin, C.-S. et al: Intracavernosal vascular endothelial growth factor (VEGF) injection and adeno-associated virus-mediated VEGF gene therapy prevent and reverse venogenic erectile dysfunction in rats. Int J Impot Res, in press, 2002
9. Duijvestijn, A. M., van Goor, H., Klatter, F., Majoor, G. D., van Bussel, E. and van Breda Vriesman, P. J.: Antibodies defining rat endothelial cells: RECA-1, a pan-endothelial cell-specific monoclonal antibody. Lab Invest, 66: 459, 1992
10. Glantz, S. A.: Primer of Biostatistics, 3rd ed. New York: McGraw-Hill, 1992
11. Mikhailidis, D. P., Khan, M. A., Milionis, H. J. and Morgan, R. J.: The treatment of hypertension in patients with erectile dysfunction. Curr Med Res Opin, suppl., 16: s31, 2000
12. Tsao, P. S. and Cooke, J. P.: Endothelial alterations in hypercholesterolemia: more than simply vasodilator dysfunction. J Cardiovasc Pharmacol, suppl., 32: S48, 1998
13. Kim, J. H., Klyachkin, M. L., Svendsen, E., Davies, M. G., Hagen, P. O. and Carson, C. C., III: Experimental hypercholesterolemia in rabbits induces cavernosal atherosclerosis with endothelial and smooth muscle cell dysfunction. J Urol, 151: 198, 1994
14. Lloyd-Jones, D. M. and Bloch, K. D.: The vascular biology of nitric oxide and its role in atherogenesis. Annu Rev Med, 47: 365, 1996
15. Jeremy, J. Y., Angelini, G. D., Khan, M. et al: Platelets, oxidant stress and erectile dysfunction: an hypothesis. Cardiovasc Res, 46: 50, 2000

The Effect of Vascular Endothelial Growth Factor and Brain Derived Neurotrophic Factor on Cavernous Nerve Regeneration in a Nerve-Crush Rat Model

Authors: Derek J. Bochinski, M.D., FRCSC, Fellow UCSF, Pao-Shiu Hsieh, M.D., Gui Tin Lin, M.D., Ph.D., Lora Nunes, Ching Shwun Lin, Ph.D., Tom F. Lue, M.D., Professor.

- All authors are from the University of California, San Francisco, Department of Urology
- Correspondence: Derek J. Bochinski, M.D.
- Email: dbochins@hotmail.com

Department of Urology

University of California

San Francisco, CA, 94143-0738

Phone (415) 476-2106

SUMMARY

Objective: To test the hypothesis that intracavernous injection of brain derived neurotrophin factor (BDNF) and vascular endothelial growth factor (VEGF) can facilitate nerve regeneration and recovery of erectile function after cavernous nerve injury.

Materials and Methods: Sprague-Dawley rats were used (n=25): 4 underwent a sham operation; 7 underwent bilateral nerve crushing with no further intervention; 14 underwent bilateral nerve crushing with either an immediate (n=7) or 1 month delayed (n=7) intracavernosal injection of BDNF and VEGF. Erectile function was assessed by cavernous nerve electrostimulation at 3 months. Neural regeneration was assessed by reduced nicotinamide adenine dinucleotide phosphate (NADPH-D) diaphorase staining and Tyrosine hydroxylase (TH) staining of penile tissue and major pelvic ganglia.

Results: After nerve crush, functional evaluation at 3 months revealed a decrease in intracavernosal pressure with cavernosal nerve stimulation (mean cavernosal pressure 33.9 cm H20 $\pm$ 15.3)(Sham group had mean intracavernosal pressures of 107.8 cm H20 $\pm$ 18.1). With immediate BDNF and VEGF injection intracavernous pressures were significantly higher then controls (67.8 cm H20 $\pm$ 38.5). Even a delayed injection of BDNF and VEGF produced an improvement in intracavernous pressures (78.0 cm H20 $\pm$ 21.8). Histological analysis of specimens subjected to NADPH-D and TH staining revealed a significant change of the morphology of terminal branches of the cavernous and dorsal nerves as well as the staining quality of the neurons in the MPG. Quantitatively when the number of positive staining nerve fibers were counted, their appeared to be a trend toward reverting back to normal numbers after treatment with immediate and delayed VEGF + BDNF.

Conclusi n: Intracavernous injection of BDNF/ VEGF appears to both prevent degeneration and facilitate regeneration of nNOS containing neurons in the major pelvic ganglia, dorsal nerve and intracavernous tissue. Therefore it may have the therapeutic potential to enhance the recovery of erectile function after radical pelvic surgeries.

Key Words: VEGF, BDNF, Impotence, Nerve Regeneration

INTRODUCTION

The anatomical location of the cavernosal nerves run posterolateral to the prostate and thus render them vulnerable to injury during radical prostatectomy or cystoprostatectomy. Various techniques have been applied to try to decrease the incidence of erectile dysfunction post-prostatectomy. Walsh (1) developed a nerve sparing technique which has reduced the incidence of erectile dysfunction postoperatively. Despite these advances erectile dysfunction still plaques a significant proportion of patients and many of these patients do not recover their erectile capacity.

In many instances during nerve sparing prostatectomy the cavernosal nerves may have been inadvertently damaged by manipulation and substantial axonal damage may have been incurred. We believe that recovery of erectile function may be dependent on resprouting of nerves from the remaining neural tissue (2). In our previous report we have shown that systemic growth hormone injection enhanced cavernous nerve regeneration in a nerve-injury rat model (3). However, there are obvious concerns regarding the use of systemic growth hormones and their inadvertent side effects.

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors that include Nerve growth factor (NGF), Neurotrophin – 3 (NT-3) and

Neurotrophin - 4 (NT-4). BDNF has been shown to enhance the survival and differentiation of several classes of neurons in vitro (4). BDNF is expressed within peripheral ganglia and not restricted to neuronal target fields. It may thus act in an autocrine or paracrine fashion on neurons (5) . Neurotrophins have a significant role in regeneration of neurons. After transection or focal crush injury there is a rapid influx of nerve growth factors to the segment distal from the site of injury (6). Interestingly BDNF is only induced in distal segments 2 weeks after injury (7). It is reasonable to assume however that these neurotrophins and their corresponding receptors play an important role in promoting neuronal survival and differentiation around the time of peripheral nerve injury.

While VEGF-A has been extensively studied for its mitogenic effects on vascular endothelium, it has also been observed to have significant musculotrophic and neurotrophic properties (8-9). It is through these pathways that VEGF-A exerts its protective effects on the cavernosal and neural tissue. VEGF-A has been found to induce the endothelial and inducible forms of nitric oxide synthase (NOS) in the penis of rats (10). NOSs are responsible for the production of nitric oxide which results in vascular smooth muscle relaxation and penile erection.

In this study we injected penile tissue with a combination of neurotrophic factors (VEGF+BDNF) after a nerve crush injury. Functional studies and immunohistochemical analysis were performed to assess the tissues response to treatment.

MATERIALS AND METHODS

Experimental Animals

25 male Sprague Dawley rats were used (3 months of age , 250-300 gm) . Rats were divided into 4 groups: 1) Sham group (n= 4) underwent midline incision and dissection of the cavernous nerves without any further surgical manipulation ; 2) Control group (n=7) underwent dissection of the cavernous nerves and subsequent intentional 2-minute crush injury with a hemostatic clamp ; 3) Immediate VEGF and BDNF group (n=7) underwent the same crush injury of the cavernous nerves followed by intracavernosal injection of VEGF and BDNF ; 4) Delayed VEGF and BDNF group (n=7) underwent the same crush injury followed 1 months later by an intra-cavernous injection of VEGF and BDNF. The erectile response was assessed in all rats at 3 months by electro-stimulation of the cavernous nerves and measurement of intracavernous pressures. In addition, preceding euthanasia samples of major pelvic ganglion and penile tissue were collected for NADPH-D and TH staining.

Surgical Procedure

Rats were anesthetized by initial induction using isoflurane inhalation followed by intraperitoneal injection of sodium pentobarbital (40 mg/kg). The rats were kept isothermic by placement on a heating pad (temperature of 37 degrees Celsius). A lower midline abdominal incision was performed after the abdomen was shaved and prepped with an iodine-based solution. The prostate gland was exposed and the cavernous nerves that were tracking posterolaterally were identified and isolated. The major pelvic ganglion (MPG) was also identified more proximally along the course of the cavernous nerve. In the sham group (group 1) no further surgical manipulation was performed. In the remaining groups the cavernosal nerves were isolated and a crush injury was performed using a hemostat clamp for 2 minutes duration. Following crush injury in the control (group 2) and delayed group (group 4) the abdomen was closed. In the immediate group (group 3) recombinant VEGF (4000 ng/rat) and BDNF (600

ng/rat) were injected into the corpus cavernosum. Abdominal closure in all rats was performed in two layers.

In the delayed group (group 4) 1 month following crush injury an intracavernous injection of VEGF and BDNF was performed. At 3 months erectile function was assessed in all rats. A repeat midline abdominal incision was performed and the cavernous nerves were exposed and isolated in all rats. The skin overlying the penis was incised and the crura of the penis were identified. A 23 gauge scalp vein needle filled with 250 U/ml of heparin solution was connected to PE-50 tubing and was inserted into the right crus body for pressure measurements. A bipolar stainless steel electrode was used to directly stimulate the cavernous nerve (probes are 2 mm in diameter and separated by 1 mm). Mono-phasic rectangular pulses were generated by a Macintosh computer with a custom built constant current amplifier. Stimulus parameters were 1.5 mA, frequency of 20 Hz, pulse width 0.2 msec, and duration of 50 seconds. The intracavernous pressures were recorded in all rats (see Figure 1) using a Macintosh computer program with Lab VIEW 4.0 software (National Instruments, Austin TX). After functional evaluations were performed a mid-shaft corporal sample was obtained as well as a sample of the MPG. NADPH and TH immuno-staining was performed on these specimens.

Preparation of BDNF and VEGF

We used a human recombinant Brain Derived Neurotrophin Factor which was expressed in *SF* 21 insect cells. The contents were reconstituted using 0.2 um filtered PBS containing 0.1% HSA or BSA to a concentration not less then 10 ug/ml.

Similarily a human recombinant VEGF expressed from *SF* 21 insect cells was used. VEGF was reconstituted using 0.2 um filtered PBS containing 0.1% HSA or BSA to a final concentration of not less then 1 ug/ml

NADPH Diaphorase and Tyrosine Hydroxylase Staining

NADPH Diaphorase and TH staining was performed on 4 random rats in each group. Tissues were fixed for 4 hours in phosphate buffer containing 0.002% picric acid and 2% formaldehyde, then transferred to 30% sucrose prior to freezing. Ten-micron serial cryosections were adhered to charged slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). After air drying for 5 minutes, the sections were incubated with 0.1 mM NADPH, 0.2 mM nitroblue tetrazolium, 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in buffer with constant microscopic monitoring for color development. When a deep blue color was detected for NADPH-diaphorase positive nerves; rinsing in buffer terminated the reaction. For TH staining, endogenous peroxidase activity was quenched by incubating slides in 3% H2O2/methanol for 10 minutes. After rinsing, slides were incubated in blocking serum followed by overnight incubation with primary antibody (1:100 Tyrosine Hydroxylase Mab, NovoCastra, Belmont, CA). After washing with buffer, sections were immunostained using the avidin-biotin-peroxidase method (Elite ABC, Vector Labs, Burlingame, CA), with diaminobenzidine as the chromagen [17].

Photography and Image Analysis

All sections were photographed using a Nikon DXM1200 digital still camera attached to a Leica Laborlux microscope, utilizing ACT-1 software (Nikon Instruments Inc., Melville NY). Some images were analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

The presence of NADPH diaphorase-positive nerves is easily apparent with this stain and is seen as a highly localized, densely blue region. The staining pattern was assessed by counting

the number of the NADPH-positive nerve fibers present in (magnification 400X) each corpus cavernosum (endothelium staining was not included in the count).

Statistical Analysis

Statistical analysis was performed using Biostat Primer (Statview 4.02 software, Macintosh). Statistical groups were compared using a Student – Newman – Keuls test. Groups were considered to be significantly different at $p<0.05$.

Results

Using a Student Newman Keuls test, comparison of data reveals a significant difference in intracavernosal pressures (see Table 1a-b) between the sham group (Figure 1) and all other groups ($p<0.05$). Comparing the mean intracavernosal pressures between the control group (Figure 2) (33.9 cm H20) and both the immediate VEGF/ BDNF and delayed VEGF/BDNF groups reveal a significant difference in pressures ($p<0.05$). No statistically significant difference was identified in intracorporal pressures between rats that had immediate intracavernosal (Figure 3) injections of VEGF/BDNF and those with delayed injections (Figure 4). Note should be made of the fact that data on only 6 rats were used in the delayed VEGF/BDNF group (1 rat prematurely died and data could not be collected).

Immunohistochemical staining was performed on the corpora cavernosum, dorsal nerve complex, and major pelvic ganglion of 4 rats in each group. Staining was performed using an NADPH and TH stains. This revealed a distinct pattern in the specimens. The number of NADPH diaphorase positive nerve fibers in the corpora cavernosa and dorsal nerve of the nerve crush group compared to the sham group was significantly decreased ($p<0.05$) (Table 2-3). No significant differences were observed in the degree of TH staining or the % NADPH ratio (Table

2a-b). Comparison of the staining patterns of the treatment groups and the nerve crush (control) groups reveals a trend such that the % NADPH rises with treatment. Our evaluation however reveals no statistically significant difference in the treatment groups and control group. Of note, several significant observations were made microscopically. After nerve crushing the microscopic appearance of the nerves in the corpora cavernosa appears quite attenuated, thinned and beaded (Figure 6). This is in contrast to the sham (Figure 5) and treatment groups (Figure 7) where the nerves have more normal morphological features. Similar observations were made in the dorsal nerve and MPG (Figures 8-13).

Discussion

There are several cavernous nerve injury models reported in the literature. One group of researchers used either a crush model with a predetermined weight or a nerve-cutting model with re-approximation of the cut ends (11-12). In our previous studies, we have used bilateral nerve freezing models to be certain that there would be minimal or no nerve recovery after 3 months [13]. In the current study, we have tried a new approach-with bilateral nerve crush using a hemostat for 2-minutes, again to be sure that there would be minimal recovery of nerve function after 3 months. We were happy to find that the degree of erectile function recovery in the current hemostat-crushed model are similar to those of the freezing-injured model. The major advantage of the hemostat-crush model is its simplicity and reproducibility among various researchers involved in the experiments.

We have previously reported the beneficial effect of adeno-associated virus mediated BDNF (AAV-BDNF) gene transfer in the freeze-injured model (13). Although there was improvement of erectile function after AAV-BDNF therapy, we felt that the approach was not

suitable for clinical application. First, only partial recovery of erectile function was noted. Second, the use of gene transfer via viral vectors remain a major concern to the public and the regulatory agencies. Third, the efficiency and duration of gene transfer is not known. Therefore, we used BDNF protein in the current experiments. Since we did not know the optimal frequency and duration of BDNF protein therapy, we decided to give either one injection immediately after nerve injury or 1 month after injury in the two treatment groups respectively. The two experiments enabled us to evaluate whether BDNF had both preventive and restorative effects on the injured cavernous nerve.

Instead of intravenous or intraperitoneal injection, we chose intracavernous injection to minimize systemic side effects and undesirable angiogenesis or neural growth from VEGF or BDNF on other tissues such as retina. Retrograde transport of BDNF from axons to the neuronal cell body has been reported in other tissues (14). Therefore, intracavernous injection theoretically could provide the best chance for the axons of the cavernous nerve to pick up the injected neurotrophin and transport it to the cell body in the major pelvic ganglion to enhance neural regeneration.

Vascular endothelial growth factor (VEGF) has also been known as vascular permeability factor (VPF) because of its effect on enhancing the permeability of the endothelium. Others have taken advantage of this and were able to show the additive angiogenic effect of giving platelet derived growth factor (PDGF) and VEGF together (15). In our in vitro studies (data not shown) we also noted that VEGF had a neurotrophic effect on the cultured major pelvic ganglia of the rats. When both VEGF and BDNF were added to the culture medium, an additive effect on neural growth was evident. Therefore, we injected both VEGF and BDNF proteins into the corpus cavernosum with the hope that VEGF would not only enhance the diffusion of BDNF

into the penile tissue to be absorbed by the axons but also had an additive effect on neural regeneration. Our pilot in vivo study did prove our hypothesis that VEGF + BDNF produces better recovery of erectile function than VEGF or BDNF alone (data not shown).

Another interesting finding is the decrease in the ratio of NOS/TH nerves after crush injury of the cavernous nerves. Our previous studies have clearly shown the decrease in NOS-containing nerves after cutting or freezing injury. The current study is unique because double staining with NADPH-D and TH can clearly demonstrate the "competitive sprouting" of the TH-containing sympathetic nerves when the NOS-containing nerve degenerate after cavernous nerve injury. Previous reports have suggested that the NOS-containing parasympathetic nerve fibers of the penis originate from the dorsal caudal portion of the major pelvic ganglion and the cavernous nerve while the TH-containing sympathetic nerve fibers originate from sympathetic chain and enter the penis via the pudendal nerve and cavernous artery (16-18). Theoretically, the overgrowth of TH-containing sympathetic nerve fibers may cause excessive contraction of penile smooth muscles and result in "shrunken penis" – a frequent complaint of patients after radical prostatectomy which selectively damages the cavernous nerves.

Besides the discovery of an altered ratio of NOS/TH nerve fibers after cavernous nerve injury, the study also shows that intracavernous injection of VEGF + BDNF protein enhances the recovery of NOS nerve fibers and thus restores the ratio of NOS/TH nerve fibers. Although the difference is not statistically significant because of the small number of animals in each group, the qualitative comparison of nerve fibers between the treated and untreated groups clearly show that VEGF + BDNF treated rats have longer and thicker nerve fibers.

Conclusion

In this study, we have shown that crush injury of the cavernous nerve with a hemostat for two minutes produces a reliable and reproducible model of neurogenic ED in the rats. Injury of the cavernous nerve results in poor erectile function and change of NOS/TH nerve ratio which may be responsible for the complaint of "shrunken penis" after radical prostatectomy. One bolus intracavernous injection of VEGF and BDNF protein immediately and 1 month after nerve injury causes a significant recovery of erectile function and the morphology of cavernous nerve fibers in the corpus cavernosum. This VEGF enhanced neurotrophin therapy may be useful for preserving and recovering of erectile function after radical pelvic surgery.

References

1. Walsh, P. C., and Mostwin, J. L.: Radical prostatectomy and cystoprostatectomy with preservation of potency. Results using a new nerve sparing technique. *Br. J. Urol.*, **56**: 694, 1984.
2. Carrier, S., Zvara, P., Nunes, L., Kour, N. W., Rehman, J., and Lue, T. F.: Regeneration of nitric oxide synthase containing nerves after cavernous nerve neurotomy in the rat. *J. Urol.* **153**: 1722, 1995.
3. Jung, G. W., Spencer, E. M., and Lue, T. F.: Growth hormone enhances regeneration of nitric oxide synthase-containing penile nerves after cavernous nerve neurotomy in rats. *J. Urol.* **160**: 1899, 1998.
4. Jones, K., Farinas, I., Backus, C., Reichardt, L.F.: Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell.* Mar 25;**76(6):**989-99. 1994.
5. Snider, W.D.: Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell.* Jun 3;**77(5)**:627-38. Review. 1994
6. Johndon, E. M., Jr., Taniuchi, M., and DiStefano, P. S.L.: Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci.* **Jul;11(7)**:299-304. 1988
7. Barde, Y. A., Edgar, D., Thoenan, H.: Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1(5)**:549-53. 1982

8. Brown, L. F., Detmar, M., Tognazzi, K., Abu-Jawdeh, G., Iruela-Arispe, M. L.: Uterine smooth muscle cells express functional receptors (flt-1 and KDR) for vascular permeability facto/vascular endothelial growth factor. *Lab Invest*: **76**; 245-255; 1997.

9. Sondell, M., Sundler, F., Kanje, M.: Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci;* **12**; 4243-4254; 2000.

10. Lin, C. S., Ho, H., Chen, K., Lin, G., Nunes, L., and Lue, T. F.: Intracavernosal injection of vascular endothelial growth factor induces nitric oxide synthase isoforms. *Br. J. of Urol.* **89**; 955-960; 2002

11. Quinlan, D. M., Nelson, R. J., Walsh, P. C.: Cavernous nerve grafts restore erectile function in denervated rats. *J. Urol.*. Feb; **145(2)**; 380-383; 1991

12. Sezen, S. F., Hoke, A., Burnett, A. L., Snyder, S. H.: Immunophilin ligand FK506 is neuroprotective for penile innervation. *Nat. Med. Oct*; **7(10)**; 1073-4; 2001

13. Bakircioglu, M. E., Lin, C. S., Fan, P. Sievert, K. D. Kan, Y. W., Lue, T. F.: The effect of adenoassociated virus mediated brain derived neurotrophic factor in an animal model of neurogenic impotence. *J. Urol.* June; **165 (6 Pt 1)**; 2103-9; 2001

14. Huang, E. J., Reichardt, L. F.: Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**; 677;2001.

15. Richardson, T. P., Peters, M. C., Ennett, A. B.: Polymeric system for dual growth factor delivery. *Nat. Biotechnol.* **19**; 1029; 2001

16. Schirar, A., Giuliano, F., Rampin, O.: A large proportion of pelvic neurons innervating the corpora cavernosa of the rat penis exhibit NADPH-diaphorase activity. *Cell Tissue Res.* **278**; 517; 1994.

17. Dail, W. G., Harji, F., Gonzales, J. et al.: Multiple vasodilator pathways from the pelvic plexus to the penis of the rat. *Int. J. Impot. Res.* **11**;277; 1999.

18. Andersson, K. E., Hedlund, P., Alm, P.: Sympathetic pathways and adrenergic innervation of the penis. *Int. J. Impot. Res.* **12**; S5; 2000.

Tables

Table 1a

Mean Intracavernosal Pressures in response to Electrostimulation

Group	Number of Rats	Intracorporal Pressure (cm H20) *
1 (Sham)	4	107.8 ± 18.1
2 (Control)	7	33.9 ± 15.3
3 (Immediate)	7	67.8 ± 38.5
4 (Delayed)	6	78.0 ± .8

*represents means and standard deviations

Table 1b

Comparison of Mean Intracavernosal Pressures

Groups	P Values
Sham vs Control	<0.05
Sham vs Immediate VEGF+BDNF	<0.05
Sham vs Delayed VEGF+BDNF	<0.05
Control vs Immediate VEGF+BDNF	<0.05
Control vs Delayed VEGF+BDNF	<0.05
Immediate vs Delayed VEGF+BDNF	>0.05

Table 2a

Corpora Cavernosum Immunohistochemical Staining

Group	Number of Rats in Group	NADPH *	Tyrosine Hydroxylase *	% NADPH **
Sham	4	90.5 ± 11.3	33.5 ± 29.2	75.2 ± 16.6
Control	4	22.8 ± 23.0	38.8 ± 24.1	37.2 ± 30.8
Immediate	4	44.3 ± 23.7	23.0 ± 14.0	61.2 ± 27.0
Delayed	4	15.3 ± 5.3	13.3 ± 10.7	59.9 ± 19.0

*mean number of nerve fibers staining positive per specimen submitted (with standard deviation)

**represents a ratio of NADPH staining fibers and TH staining fibers

Table 2b

Comparison of Mean %NADPH in Corpora Cavernosa

Groups	P value
Sham vs Control	=0.07
Sham vs Immediate VEGF+BDNF	=0.43
Sham vs Delayed VEGF+BDNF	=0.27
Control vs Immediate VEGF+BDNF	=0.28
Control vs Delayed VEGF+BDNF	=0.25
Immediate vs Delayed VEGF+BDNF	=0.92

Table 3a

Dorsal Nerve Immunohistochemical Staining

Group	Number of Rats in Group	NADPH *	Tyrosine Hydroxylase *	% NADPH**
Sham	4	71.6 ± 41.2	40.8 ± 38.7	67.4 ± 14.8
Control	4	18.2 ± 20.0	55.7 ± 33.8	21.3 ± 17.8
Immediate	4	28.8 ± 16.8	73.9 ± 35.2	27.6 ± 11.3
Delayed	4	34.2 ± 19.8	64.0 ± 39.1	36.1 ± 14.9

*mean number of nerve fibers staining positive per specimen submitted (with standard deviation)

** represents a ratio of NADPH staining fibers and TH staining fibers

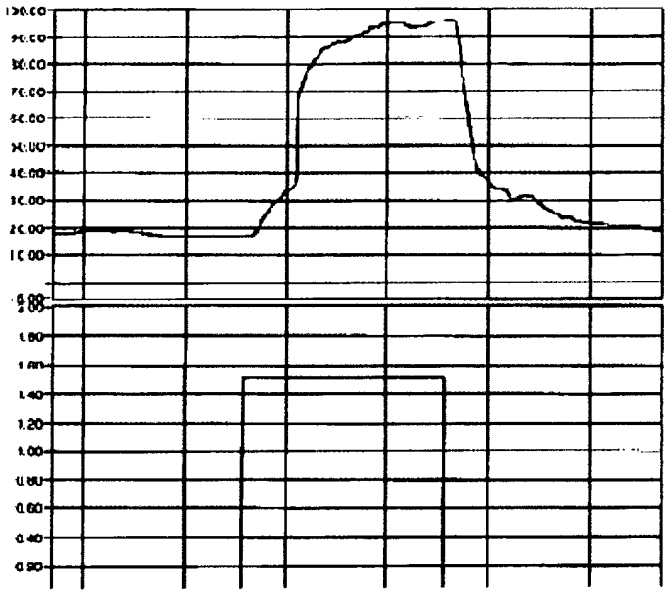
Table 3b

Comparison of Mean %NADPH in Dorsal Nerve

Groups	P Values
Sham vs Control	=0.07
Sham vs Immediate VEGF+BDNF	=0.01
Sham vs Delayed VEGF+BDNF	=0.03
Control vs Immediate VEGF+BDNF	=0.57
Control vs Delayed VEGF+BDNF	=0.25
Immediate vs Delayed VEGF+BDNF	=0.40

Figures

Fig 1. Sample Measurements of Cavernosal Pressures During Electrostimulation (Group 1-Sham Group)

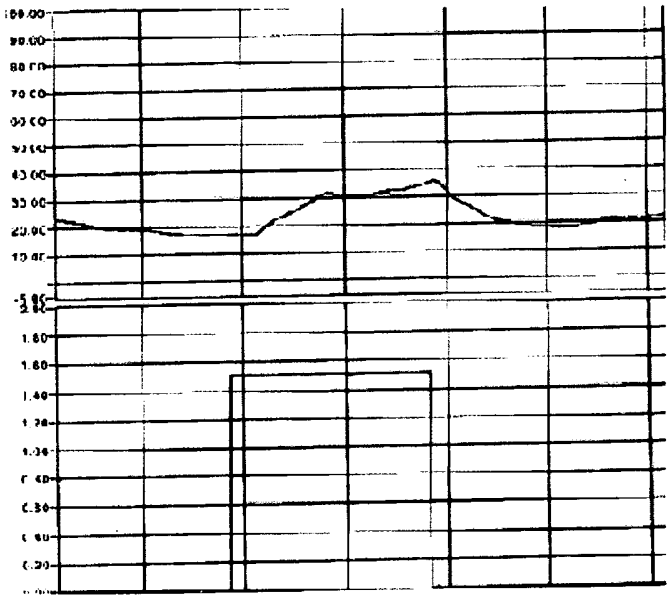


19. Upper Y axis represents intracavernosal pressures in cm H20

20. Lower Y axis represents amplitude of current from electrode in milliamps

21. X axis represents time in seconds (area under rectangle is 50 seconds)

Figure 2-. Sample Measurements of Cavernosal Pressures During Electrostimulation (Group 2- Crush Model)



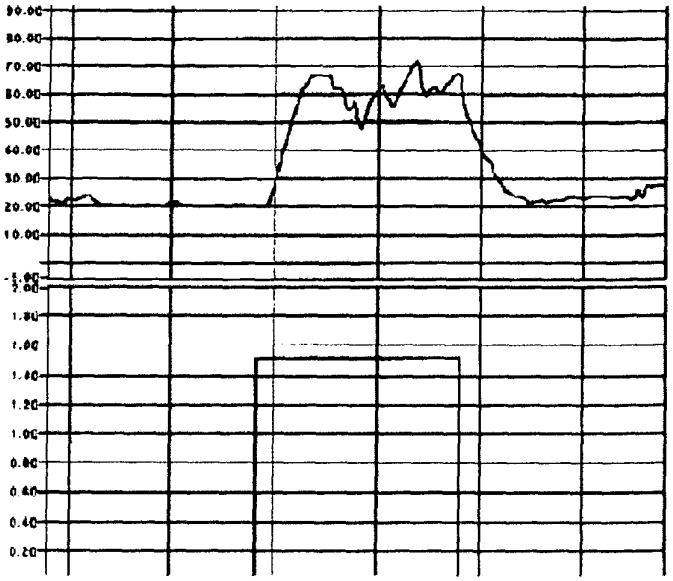
22. Upper Y axis represents intracavernosal pressures in cm H20

23. Lower Y axis represents amplitude of current from electrode in milliamps

24. X axis represents time in seconds (area under rectangle is 50 seconds)

Figure 3- Sample Measurements of Cavernosal Pressures During Electrostimulation

(Group 3 – Immediate VEGF and BDNF)

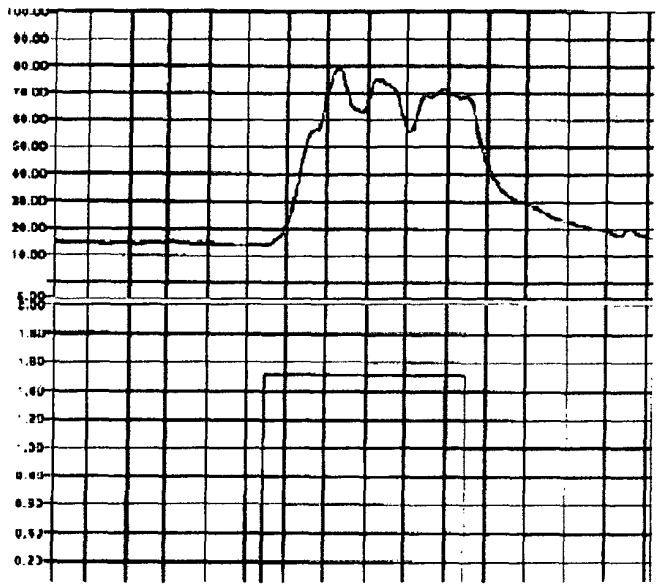


25. Upper Y axis represents intracavernosal pressures in cm H20

26. Lower Y axis represents amplitude of current from electrode in milliamps

27. X axis represents time in seconds (area under rectangle is 50 seconds)

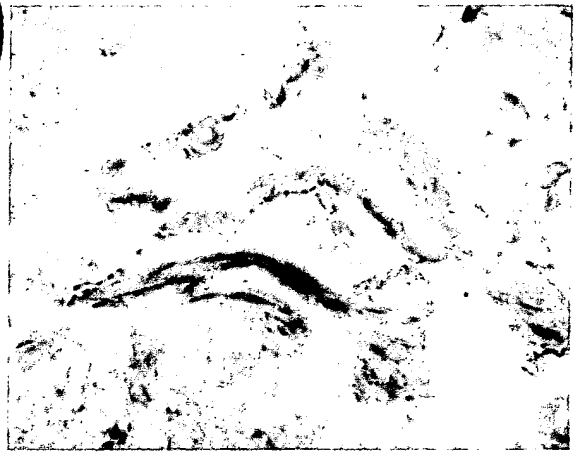
Figure 4- Sample Measurements of Cavernosal Pressures During Electrostimulation (Group 4 – Delayed VEGF and BDNF)



28. Upper Y axis represents intracavernosal pressures in cm H20

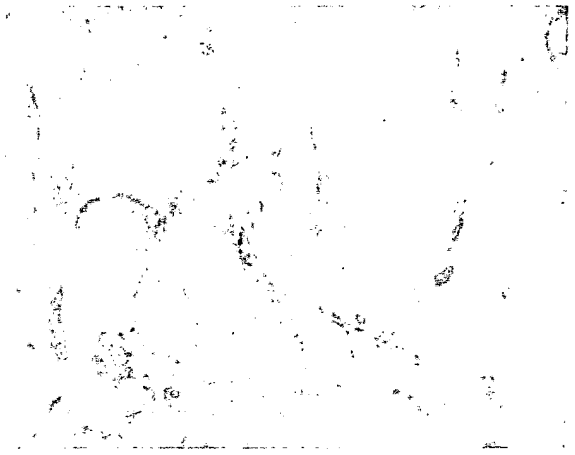
29. Lower Y axis represents amplitude of current from electrode in milliamps

30. X axis represents time in seconds (area under rectangle is 50 seconds)



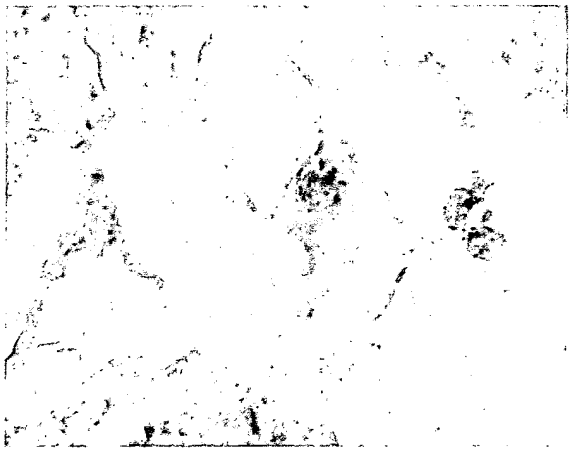
Sham

Figure 5



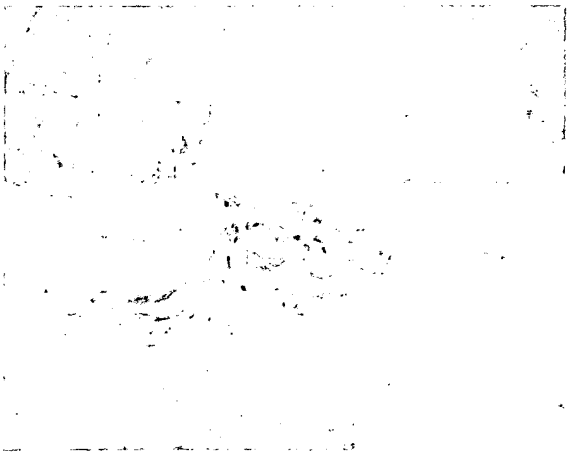
Crush

Figure 6



Immediate VEGF + VEGF

Figure 7

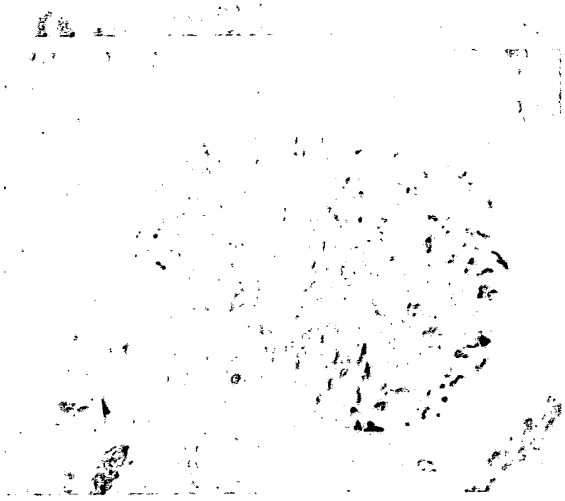


1 Month VEGF + BDNF

not included

Sham

Figure 8

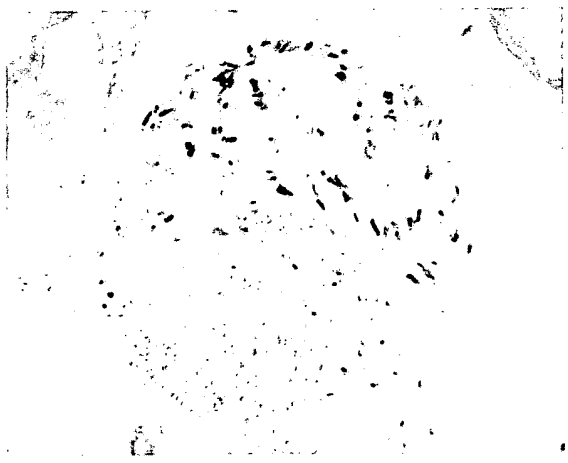


Crush

Figure 9

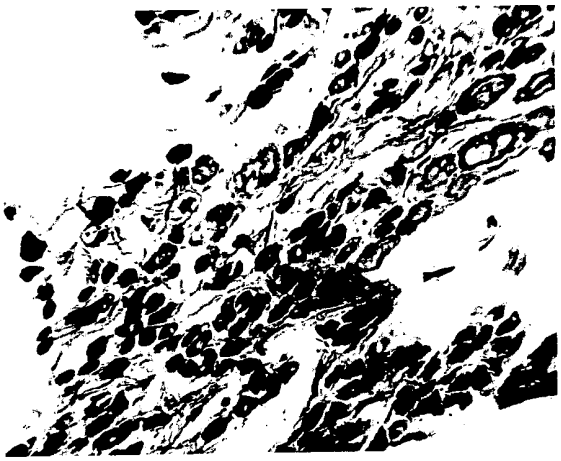
Immediate VEGF + BDNF

not included



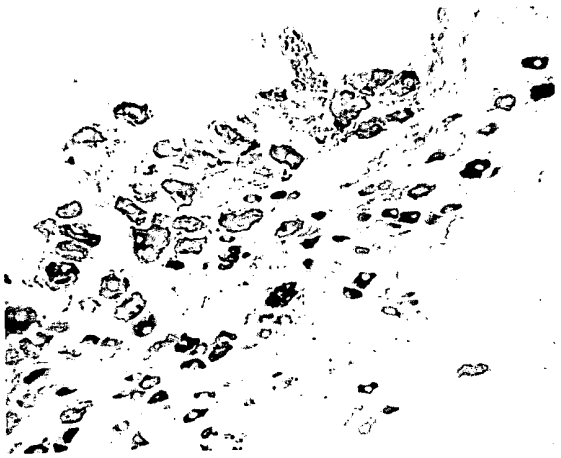
1 Month VEGF + BDNF

Figure 10



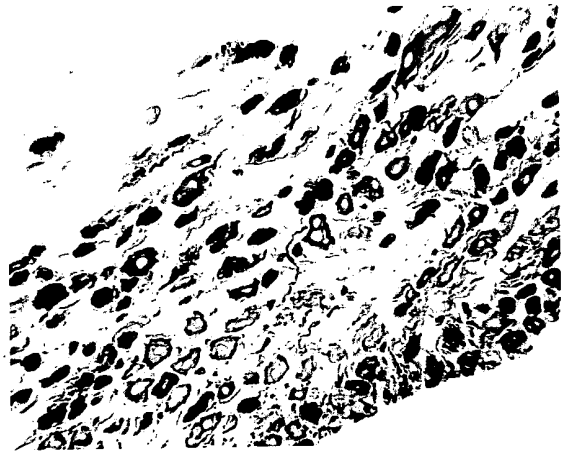
Sham

Figure 11



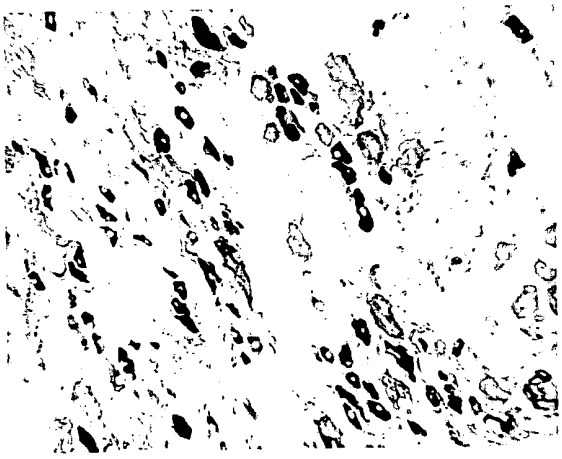
Crush

Figure 12



Immediate VEGF + BDNF

not included



1 Month VEGF + BDNF

Figure 13

Legends

Figure 1. Electrostimulation of the cavernous nerves at 12 weeks in the sham group. Note the peak intracavernosal pressure reaches 96 cm H20.

Figure 2. Electrostimulation of the cavernous nerves at 12 weeks in the control group. Note the peak intracavernosal pressures are significantly reduced to approximately 35 cm H20.

Figure 3. Sample tracing of electrostimulation of the cavernous nerves at 12 weeks in immediate VEGF and BDNF group. Intracavernosal pressures have recovered such that a peak pressure of 72 cm H20 is reached

Figure 4. Sample tracing of electrostimulation of the cavernous nerves at 12 weeks in the delayed VEGF and BDNF group. The peak intracavernosal pressures in this example reach 80 cm H20.

Figure 5. NADPH diaphorase and TH staining of intracavernous erectile tissue in sham group. Note the relative abundance of blue staining nerve fibers (tailed arrows) that represent nNOS nerve fibers. The brown staining nerve fibers represent TH staining (tail-less arrow) and sympathetic fibers (Magnification 400 X)

Figure 6. NADPH diaphorase and TH staining of intracavernous erectile tissue in nerve crush (control) group. Note the absence of NADPH staining that is represented by blue staining, and the dominance of brown TH staining (tail-less arrow). (Magnification 400 X)

Figure 7. NADPH diaphorase and TH staining of intracavernous erectile tissue in immediate VEGF and BDNF treatment group. Increase in intracavernosal pressures with stimulation is present along with an increase in NADPH staining nerve fibers (arrows). (Magnification 400 X)

Figure 8. NADPH diaphorase and TH staining of dorsal nerves in sham group. Note the predominantly blue staining of nerve fibers representing parasympathetic nerves (tailed arrows). (Magnification 400 X)

Figure 9. NADPH diaphorase and TH staining of dorsal nerves in control group. Note the relative paucity of blue staining fibers and predominant brown stain (tail-less arrows). (Magnification 400 X)

Figure 10. NADPH diaphorase and TH staining of dorsal nerves in nerve crush model after delayed treatment with intracavernous VEGF and BDNF. A relative increase in blue staining (NADPH) nerve fibers is seen relative to Figure 9 (tailed arrows). (Magnification 400 X).

Figure 11. NADPH diaphorase and TH double staining of major pelvic ganglia in sham group. Note the dense staining of parasympathetic nerve fibers in blue (tailed arrows). (Magnification 400 X)

Figure 12. NADPH diaphorase and TH double staining of major pelvic ganglia in control group. After nerve crush there is a decrease in staining of parasympathetic nerves (tailed arrows) and preservation of staining quality of sympathetic nerves (represented by the brown stain) (Magnification 400 X)

Figure 13. NADPH diaphorase and TH double staining of major pelvic ganglia after nerve crush and delayed intracavernosal injection of VEGF and BDNF. An increased number of nerve fibers (arrows) stain positive with the NADPH stain.(tailed arrows) (Magnification 400 X)